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Indole-3-acetic acid biosensor based on G-rich DNA labeled AuNPs as chemiluminescence probe coupling the DNA signal amplification

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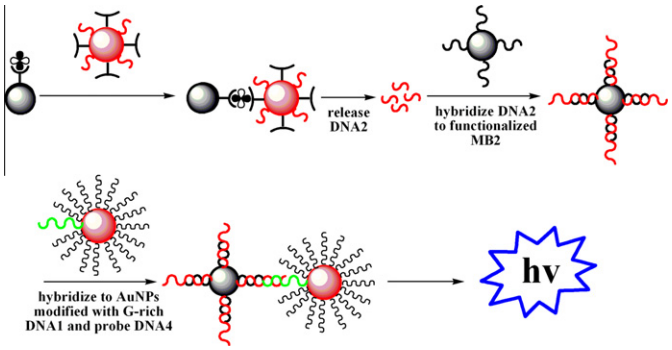
HIGHLIGHTS

- The G-rich DNA labeled gold nanoparticles used as CL probe.
- The CL reaction between TMPG and G-rich DNA used for IAA detection.
- The limit of detection is 0.01 ng/mL for IAA.

GRAPHICAL ABSTRACT

The detection signal is amplified through G-rich DNA labeled AuNPs as CL probe and released DNA which further acts as a linker. The signal amplification technology provides a detection limit of 0.01 ng/mL for IAA.



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ABSTRACT

A highly sensitive chemiluminescence (CL) method for detection of phytohormone indole-3-acetic acid (IAA) was developed by using G-rich DNA labeled gold nanoparticles (AuNPs) as CL probe coupling the DNA signal amplification technology. The IAA antibody was immobilized on carboxyl terminated magnetic beads (MBs). In the presence of IAA, antibody labeled AuNPs were captured by antibody functionalized MBs. The DNA on AuNPs is released by a ligand exchange process induced by the addition of DTT. The released DNA is then acted as the linker and hybridized with the capture DNA on MBs and probe DNA on AuNPs CL probe. The CL signal is obtained via the instantaneous derivatization reaction between a specific CL reagent, 3,4,5-trimethoxyl-phenylglyoxal (TMPG), and the G-rich DNA on AuNPs CL probe. IAA can be detected in the concentration range from 0.02 ng/mL to 30 ng/mL, and the limit of detection is 0.01 ng/mL.

Introduction

Phytohormones play very important roles at almost all developmental stages of plants [1]. The content of a phytohormone in a plant is usually very low. For instance, the tissue concentration for phytohormones usually ranges from 10 to 100 ng/g for

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indole-3-acetic acid (IAA), from 1 to 100 ng/g for gibberellins (GAs), from 1 to 100 ng/g for cytokinins (CKs), and from 10 to 50 ng/g for abscisic acid (ABA) [2–4]. Because of their extremely low concentrations in plant tissues and they are highly sensitive to environmental factors such as light, heat and oxygen, plant physiologists urgently require a sensitive, precise, and convenient measurement procedure for phytohormone assay in plants. Traditional phytohormone analysis techniques such as gas chromatography [5,6], gas chromatography/mass spectrometry [7], liquid chromatography/mass spectrometry [8], capillary electrophoresis [9], high-performance liquid chromatography (HPLC) [10,11], enzyme-linked immunosorbent assay [12], liquid chromatography–tandem mass spectrometry [13] and impedance immunosensor [2,3] have been broadly reported.

In high sensitive biosamples detection technology, many improvements have been made by using nanoparticles (NPs) and magnetic beads (MBs) in order to enhance sensitivity and reduce detection time in recent years [14,15]. NPs such as gold NPs (AuNPs), as a class of nanomaterials with many unique properties such as colorimetric, conductivity, and nonlinear optical properties [16,17], have found many successful applications in biomolecular detection [18–20]. AuNPs stand out from a variety of NPs used in bioassays because of their biocompatibility, rapid and easy preparation, narrow size distribution and efficient coating by thiols or other bioligands [20]. MBs are quite fascinating because of their diverse uses, including biochemical applications involving separation and purification protocols and biomolecular immobilizing. MBs, as a special biomolecular immobilizing carrier, have been widely applied in immunoassays; enzyme, DNA and protein immobilization and purification; and magnetically controlled transport of anticancer drugs [21–23]. An analytical method using MB has many advantages in that they have potentially larger surface area which may provide the increased efficiency of biomolecules immobilization, and target antigen in clinical or human serum samples can be directly captured, easily separated and even concentrated by antibody-coated MBs, and analyzed by immunoassay [24]. Now, many novel ultrasensitive detection systems have been developed based on MBs [15,25,26].

Up to now, some methods have been developed by coupling the advantageous properties of AuNPs and MBs in immunoassays [15,16,27–29]. In this contribution, we constructed a new chemiluminescent (CL) immunoassay protocol for the detection of IAA using the G-rich DNA labeled AuNPs as CL probe coupling the DNA signal amplification along with CL signal produced from instantaneous derivatization reaction between 3,4,5-trimethoxylphenylglyoxal (TMPG) and the G-rich DNA on AuNPs CL probe. The detection limit of 0.01 ng/mL for IAA is obtained.

Experimental

Reagent and instruments

All chemicals used were of analytical grade and were used as received. All solutions were prepared with deionized and autoclaved water from a Millipore system. 3,4,5-Trimethxylphenylglyoxal (TMPG) kindly was donated by Prof. Jianzhong Lu and Masaaki Kai (Prof. Lu: School of Pharmacy, Fudan University, China; Prof. Kai: School of Pharmaceutical Sciences, Nagasaki University, Japan). $HAuCl_4{\cdot}4H_2O$ and dithiothreitol (DTT) were obtained from Guoyao Chemical Company (Guoyao, China). Tri(2-carboxyethyl)phosphine hydrochloride (TCEP, 98%), indole-3-acetic acid (IAA), gibberellins (GAs), cytokinins (CKs) and abscisic acid (ABA) were purchased from Alfa Aesar (Massachusetts, USA). Mouse monoclonal antibody against IAA was purchased from Creative Biomart (NY, USA), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, USA). Carboxyl groups modified magnetic nanoparticles (carboxyl modified MBs) (0.5 μm, 10 mg/mL) were purchased from Baseline Chromtech Research Centre (Tianjin, China).

Oligonucleotides were obtained from SBS Genetech Co. Ltd. (Beijing, China). Their sequences were presented in following:

DNA1, 5′-SH-(GGA GGT G)$_n$-3′ (G-rich DNA, thioled on AuNPs acting as signal probe, n = 1, 2, 3, 4, 5, 6);
DNA2, 5′-TGT TAC GAC TT CCT GAA CGT ACG-NH_2-3′ (Aminated loaded on MBs);
DNA3, 5′-NH_2-CGG CGT ACG TTC AGG-3′ (Aminated loaded on MB, and partly complementary to DNA2);
DNA4, 5′-GTC GCA GTA ACA CCC TAC GCT GCT-3′ (Thioled on AuNPs, and partly complementary to DNA2).

CL emission was detected with a FI-CL instrument (IFFM-E, Remex Analytical Instrument Co. Ltd., Xian, China). A TGL-16G centrifuge (Shanghai Anting Science Instrument Co., China) was used for centrifugation.

Preparation of 30 nm AuNPs

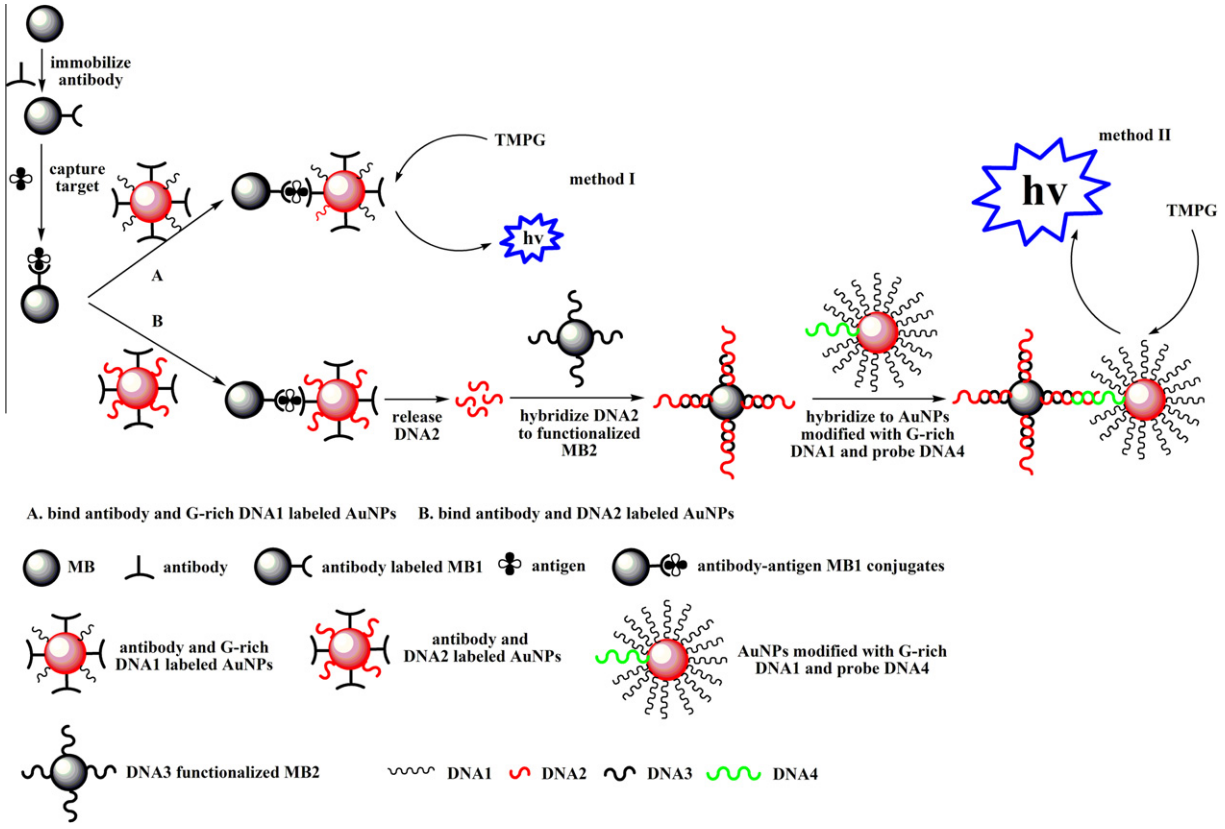
Thirty nanometer of AuNPs were prepared according to the method reported previously with a slight modification [30]. $HAuCl_4$ and trisodium citrate solutions were filtered through a 0.22 μm microporous membrane filter prior to use, and then 1.2 mL of 1% trisodium citrate was added to 100.0 mL of boiling 0.01% $HAuCl_4$ solution and stirred for 10 min at the boiling point. The final AuNPs prepared by this method have an average diameter of approximately 30 ± 3 nm as measured by TEM. The prepared 30 nm AuNPs were stored in brown glass bottles at 4 °C.

Preparation of antibody or DNA labeled MBs

The antibody was immobilized on the MBs according to the specific protocol provided by Zhang [31]. Briefly, 0.2 mL suspension of carboxylated MBs (10 mg/mL) was placed in a 5.0 mL Eppendorf tube (EP tube) and separated from the solution on a magnetic rack. The MBs were washed three times with 0.4 mL of 0.1 M imidazol–HCl buffer (pH 7.0) and then suspended to a final volume of 0.4 mL in the same buffer solution. Then 0.25 M NHS and 0.5 M EDC (0.4 mL) were added to the EP tube and the mixture was incubated at 37 °C for 30 min to activate the carboxylate groups on the MBs, followed by washing three times with 0.4 mL of 0.1 M PBS and resuspending to a final volume of 0.2 mL. And it was divided into two portions. 10 μg antibody was then added to the one portion of above freshly activated MBs solution and the resulting suspensions were allowed to stand 2 h at 37 °C for the immobilization of the antibody on the surface of the activated MBs. Finally, the resulting antibody labeled MB1 was separated from the solution magnetically and resuspended in 1% BSA solution (0.4 mL) for 2 h to eliminate the unspecific binding, and then washed twice with the above buffer solution (0.4 mL) and resuspended in 2.0 mL PBS and stored at 4 °C for further use. And the other portion of activated MBs was incubated with 0.2 mL of 1.0×10^{-7} M DNA3 at 37 °C for 2 h. Finally, the DNA3 labeled MB2 was washed with 0.2 mL of 0.1 M PBS for three times, and resuspended in 2.0 mL PBS and stored at 4 °C for further use.

Preparation of antibody and DNA labeled AuNPs

The following procedure details the preparation antibody and DNA labeled AuNPs. 10.0 mL of 1.0×10^{-6} M HS–$(CH_2)_2$–COOH solution and 0.1 mL of 10 mM TCEP were mixed at room tempera-



Scheme 1. The schematic illustration of the determination of IAA based on antibody and G-rich DNA labeled AuNPs as CL probe (method I), and G-rich DNA labeled AuNPs as CL probe coupling the DNA signal amplification technology (method II).

ture. After 1 h, 100.0 mL of 30 nm AuNPs solution was added and incubated overnight at room temperature. After centrifugation, the AuNPs were dispersed in 0.1 M PBS. Then 0.25 M NHS and 0.5 M EDC (0.4 mL) were added and incubated 1 h at 37 °C with shaking. 10 μg antibody was added and incubated at room temperature overnight. The resulting production was washed with 1.0 mL of 0.1 M PBS for three times (32). Finally, a 0.2 mL 1.0×10^{-7} M of DNA2 (step B in Scheme 1, for the preparation of antibody and DNA2 labeled AuNPs. As for the preparation of antibody and DNA1 labeled AuNPs (step A in Scheme 1), 0.2 mL 1.0×10^{-7} M of DNA1 was used) was added to the above solution, and incubated at 37 °C overnight. The resulting antibody and DNA labeled AuNPs were washed with 2.0 mL of 0.1 M PBS for three times, and resuspended in 2.0 mL PBS and stored at 4 °C for further use.

Preparation of G-rich DNA and probe DNA labeled AuNPs

The G-rich DNA and probe DNA labeled AuNPs were synthesized by adding 0.1 mL DNA1 and DNA4 aqueous to AuNPs solution. Thiol-modified DNA1 and DNA4 were activated with TCEP (10 mM) for 1 h. After shaking gently for 16 h at room temperature, the G-rich DNA1 and probe DNA4 labeled AuNPs were "aged" in the solution (0.3 M NaCl, 10 mM Tris–acetate, pH 8.2) for another 48 h. Excess reagents were removed by centrifuging at 15,000 rpm for 30 min. Following removal of the supernatant, the resulting G-rich DNA1 and probe DNA4 labeled AuNPs were washed with 0.2 mL of 0.1 M pH 7.0 PBS containing 0.1 M NaCl, recentrifuged, and then redispersed in 0.2 mL of 0.1 M pH 7.0 PBS containing 0.3 M NaCl.

Fabrication of G-rich DNA labeled AuNPs coupling DNA signal amplification biosensor

The procedure of the fabrication of G-rich DNA labeled AuNPs coupling DNA signal amplification biosensor is illustrated in Scheme 1. Briefly, IAA sample was added to antibody labeled MB1 solution. After incubated at 37 °C about 30 min, the mixture was separated with a magnetic rack. After redispersed in PBS, the antibody and DNA2 labeled AuNPs was added [13]. 30 min later, the mixture was separated with a magnetic rack and redispersed in 0.2 mL of 0.5 M DTT in 0.5 M NaCl, 0.01% Tween 20, 10 mM PBS (pH 7.2). The DTT-induced release of DNA2 was allowed to proceed for 10 min at 50 °C and 50 min at 25 °C under vortex. Magnetic field introduction removed all MBs from the solution, leaving DNA2 for hybridization with the probe DNA4 on G-rich DNA1 labeled AuNPs and capture DNA2 on MB2. After standing for 1 h at 37 °C and magnetic separation, it was taken for CL detection (method II). As for method I, after IAA sample being added to antibody labeled MB1 solution and being incubated at 37 °C about 30 min, the mixture was separated with a magnetic rack. After redispersed in PBS, the antibody and DNA1 labeled AuNPs was added at 37 °C. 30 min later, the mixture was separated with a magnetic rack and it was taken for CL detection without other procedure in method II.

CL measurements

The CL detection was carried out with a IFFS-E multifunction CL analyzer. 10 μL of tetrabutylammonium hydroxide-PBS (pH 8.5) was added into the tube. The magnetic separation products were

added. Following this, the CL reaction was triggered by injecting 100 μL of TMPG (30 mM in DMF) with a syringe through a septum after the CL analyzer began to record at 10 s [32]. The kinetics of CL signals between 0 and 30 s was recorded. The peak heights of the emission curves were measured by means of a photon counting unit.

Results and discussion

Fabrication of biosensor and detection process

G-rich DNA labeled AuNPs as CL probe coupling the DNA signal amplification IAA biosensor was employed in our design (Scheme 1). Prior to the binding of IAA, the carboxyl terminated MBs are functionalized by antibody. As a result, the antigen in the sample was first captured by the immobilized antibody on the surface of MBs, and then recognized by the antibody labeled with AuNPs which modified with antibody and DNA2. The DNA2 are released by a ligand exchange process induced by the addition of DTT. The DNA2 are then hybridized with the DNA3 on MB2 and probe DNA4 on AuNPs which modified with probe DNA and G-rich DNA. The "sandwich-type" complex was separated from the dissociative AuNPs. Since a single MB is loaded with hundreds of DNA2 strands and AuNPs CL probe modified with hundreds of G-rich DNA1, this offers a significant amplification for the detection of target IAA. In contrast, in method I, sandwich complex was formed without DNA signal amplification. After that, TMPG reacts with guanine nucleobases of DNA1 strands to CL signal. By employing this strategy, we demonstrate that this G-rich DNA labeled AuNPs as CL probe coupling the DNA signal amplification can sensitively detect 0.01 ng/mL target IAA and give about 150-fold improvement in detection sensitivity compared to nonamplified method (we choose the lowest concentration of the linear range as the comparison standard).

Optimization of experimental conditions

In order to obtain a higher sensitivity of the G-rich DNA labeled AuNPs as CL probe coupling the DNA signal amplification CL biosensor, the relative experiment conditions such as the dose of DNA3 modified MB2, the ratio of DNA1 to DNA4 for the preparation of AuNPs CL probe and the number of G bases in DNA1.

First, as shown in Fig. 1A, the CL intensity was enhanced significantly when the DNA3 modified MB2 volume was increased from 2 to 10 μL. However, at dose >12 μL the CL intensity became more flattened. Because the assay reached saturation with 12 μL DNA3 modified MB2, at this concentration, the assay response was increased slowly. Thus, there existed an optimum of DNA3 modified MB2 dose at about 12 μL DNA3 modified MB2, resulting in the best assay response. Therefore, this ration was chosen for the subsequent work.

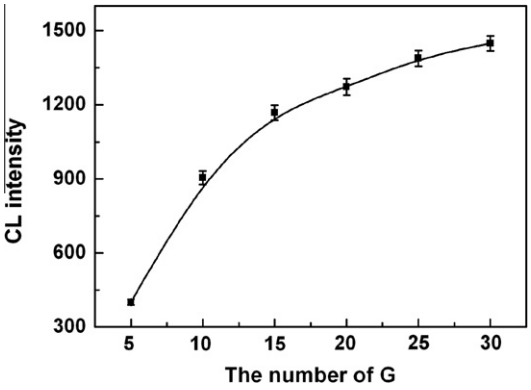


Fig. 2. The effect of number of G on CL intensity.

The ratio of DNA1 to DNA4 is significantly effect the amount of signal DNA1 and the density of probe DNA4. The effects of the ratio of DNA1 to DNA4 were subsequently examined and optimized. The CL intensity increased from 5:95, 10:90, 15:85 of ratio of DNA1 to DNA4 and then the ration increased the CL intensity decreased (Fig. 1B). Considering the decrease of the CL intensity at ratio larger than 20:80, these results suggest that the number of probe DNA4 on AuNPs reached relatively appropriate quantity. At this ratio the AuNPs can be captured relatively more by the released DNA2. At the same time, there is appropriate signal G-rich DNA1 on AuNPs. So the higher CL intensity was obtained. When the ratio is larger than 20:80, the crossreaction is increased [33] and the amount of signal DNA1 is decreased. So the CL intensity decreased. Thus, 20:80 of ratio of DNA1 to DNA4 were selected in further studies.

Dependence of CL intensity on the number of G bases was also investigated. Because the signaling mechanism is based on an instantaneous derivatization reaction between the CL reagent TMPG and G base on the AuNPs surface, the CL intensity is significantly affected by the number of G bases. Hence, increasing the number of G nucleotide on the DNA1 could enhance the CL intensity, thus five different numbers of G bases were designed and investigated in the following experiments. One can predict that increasing the number of the G base the CL intensity would increase. The experimental results suggested that CL intensity increases with increasing the number of the G base (Fig. 2). Higher CL intensity requires further increasing the number of the G base [32]. But we also found that the CL intensity increased gently when the number of the G base is more than 30. Therefore, 30 G bases of

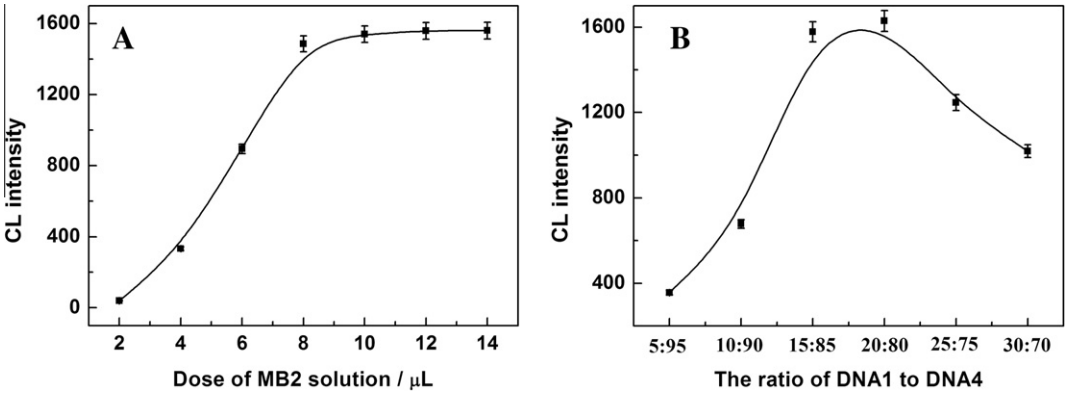


Fig. 1. The effect of dose of MB2 (A) and the ratio of DNA1 to DNA4 (B) on CL intensity.

DNA was selected in this work for economy and practicality. The stability of DNA was also studied. The experimental results suggested that CL intensity did not changed after the DNA stored 2 months at 4 °C. It showed that the DNA has good stability.

The calibration curve of IAA detection

Under the optimal experimental conditions, a nonlinear function for target phytohormone IAA was achieved from 0.02 ng/mL to 30 ng/mL with the equation of $I_{CL} = -6202.32 * \exp(-C/6.10) - 628.69 * \exp(-C/0.55) + 6795.55$ (I_{CL} is the CL intensity; C is the concentration of IAA, ng/mL; $n = 7$, $R^2 = 0.9989$). The linear range for target phytohormone IAA was 0.02 ng/mL ~ 0.3 ng/mL with the equation of $I_{CL} = 1594.70C - 3.49$ (I_{CL} is the CL intensity; C is the concentration of IAA, ng/mL; $n = 7$, $R^2 = 0.9994$) and the detection limit of 0.01 ng/mL IAA estimated using 3σ (Fig. 3A). This assay technique is an attractive alternative due to features such as high sensitivity, low cost, accessible biosensor and simple manipulation, and has the potential to measure other analytes when corresponding antibodys are used [33]. Therefore, this simple technique will offer an new CL biosensors for the sensitive and selective detection of a wide spectrum of analytes.

A control experiment without employing DNA signal amplification technology (antibody and G-rich DNA1 labeled AuNPs as CL probe, method I) was carried out to further determine the sensitivity of the proposed phytohormone assay strategy. Only antibody and G-rich DNA1 labeled AuNPs was used for the CL probe in the control experiment. The detection limit of method I is 3 ng/mL. The results indicated that G-rich DNA1 labeled AuNPs as CL probe coupling the DNA signal amplification technology (method II) giving approximately a 150-fold improvement in detection sensitivity compared to antibody and G-rich DNA1 labeled AuNPs as CL probe technology (method I) (we choose the lowest concentration of the linear range as the comparison standard) (Fig. 3B).

Selectivity of the phytohormone biosensor

Since detailed knowledge of biosensor specificity is of crucial importance for the reliability of the assays, several experiments were carried out to gain unequivocal proof of biosensor specificity. To assess the specificity of the method for the detection of IAA, experiments were conducted on phytohormone relative substance such as GAs, CKs, ABA. Different CL signals of the proposed system for the detection of 100.0 ng/mL GAs, CKs, ABA, were under the same experimental conditions according to the protocol described in experimental section, respectively. On the basis of G-rich DNA1 labeled AuNPs as CL probe coupling the DNA signal amplification technology for the detection of phytohormone molecules, GAs, CKs or ABA did not induce any significant changes in the CL signal as compared to that of IAA, which suggests that the developed strategy has a sufficient selectivity and IAA could be unequivocally identified (Fig. 4). This may be explained by the fact that the interaction between IAA antigen and IAA antibody is specific. There is no interfere when the concentration of GAs, CKs, ABA ware 100.0 ng/mL and concentration of IAA is 0.1 ng/mL (data not shown). The non-specificity interaction do not existed between IAA antibody and GAs, CKs or ABA.

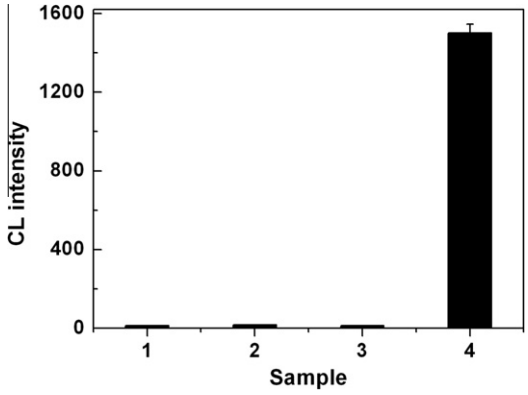


Fig. 4. CL response for different phytohormone relative substance, GAs, CKs or ABA. (1) GAs; (2) CKs; (3) ABA; (4) IAA. The concentrations were 100.0 ng/mL for GAs, CKs or ABA and 1.0 ng/mL for IAA, respectively.

Determination of IAA in a leaf of mung bean sprouts extract

To test the validation of the platform for real-world samples, analysis of *IAA* from leaf of mung bean sprouts extract were implemented. Fresh samples from mung bean sprouts were frozen in liquid nitrogen and immediately lyophilized for 24 h. To prevent light exposure, the samples were kept in the dark throughout the process. Each sample (1.0 g) was ground in a porcelain mill, passed through a 40-mesh screen, then collected in a vessel and mixed with 15 mL of 80% (v/v) methanol for overnight extraction in a refrigerator. The supernatant was collected after centrifugation at 15,000 rpm for 20 min. Finally the samples were used for IAA assay. The results are listed in Table 1. The accuracy of the assay was tested by performing the analytical recovery experiment (see the Supporting information). The results showed the feasibility of using this method for the analysis of plant samples.

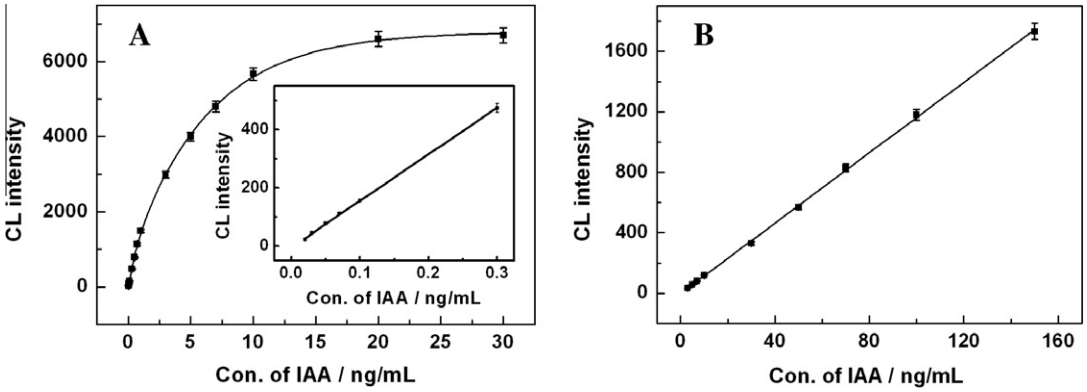


Fig. 3. Calibration curve of CL intensity vs the concentration of IAA from 0.02 ng/mL to 30.0 ng/mL (method II) (A); from 3.0 ng/mL to 150 ng/mL (method I) (B).

Table 1
Results of real samples analysis.

Sample no.	Original (ng/mL)	Added (ng/mL)	Found (ng/mL)[a]	RSD (%)	Recovery ratio (%)[b]
1	17.8	10.0	28.3	3.4	105.0
2	9.6	5.0	14.5	2.7	98.0
3	6.4	5.0	11.7	2.6	106.0

[a] Average of five determinations.
[b] Recovery(%) = (detected concentration − background content)/added concentration × 100%.

Conclusions

In conclusion, a new strategy for the simple and sensitive detection of phytohormone IAA based on G-rich DNA labeled AuNPs as CL probe coupling the DNA signal amplification technology was developed. The detection limit of 0.01 ng/mL of IAA was obtained. The biosensor exhibited high sensitivity and good selectivity as a promise alternative technique for other bioassays. This strategy could lead to the development of efficient tools for rapid, very specific and direct detection of extremely low concentration IAA. With the concept of G-rich DNA labeled AuNPs as CL probe and DNA signal amplification technology, the detection sensitivity was improved greatly. It is believable that it could make a significant contribution to phytohormone, nucleic acid, biological small molecules and protein detection based on the proof-of-concept approach described in this work.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.saa.2012.04.085.

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